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FLUORESCENCE IN SITU HYBRIDIZATION WITH HUMAN CHROMOSOME-SPECIFIC LIBRARIES: DETECTION OF TRISOMY 21 AND TRANSLOCATIONS OF CHROMOSOME 4

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Fluorescence *in situ* hybridization with human chromosome-specific libraries: Detection of Trisomy 21 and translocations of chromosome 4

(*Key Terms:* tumor cytogenetics/
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ABSTRACT

Chromosomes can be specifically stained in metaphase spreads and interphase nuclei by *in situ* hybridization with entire chromosome-specific DNA libraries. Unlabeled human genomic DNA is used to inhibit the hybridization of sequences in the library that bind to multiple chromosomes. The target chromosome can be made at least 20 times brighter per unit length than the others. Trisomy 21 and translocations involving chromosome 4 can be detected in metaphase spreads and interphase nuclei using this technique.

INTRODUCTION

The application of non-radioactive *in situ* hybridization with chromosome-specific probes for cytogenetic analysis has increased significantly in recent years. This has been due to the technical convenience of these procedures (1-3) and to the increased availability of probes that hybridize intensely and specifically to selected chromosomes. Such probes are now available for over half of the human chromosomes. In general, these bind to repeated sequences on compact regions of the target chromosome near the centromere. However, one probe has been reported that hybridizes to human chromosome 1p36, and there are several probes that hybridize to human chromosome Yq (4 and citations therein). Hybridization with these probes permits rapid identification of chromosomes in metaphase spreads, determination of the number of copies of selected chromosomes in interphase nuclei(5-7), and determination of the relative positions of chromosomes in interphase nuclei(4-6,8,9-11). Interphase analysis is especially promising in clinical applications where rapid results are desirable (eg. in prenatal diagnosis), where cells are difficult to culture *in vitro*, or where concern exists about the ability to stimulate a representative fraction

of cells into proliferation for metaphase analysis (eg. in tumor studies).

However, many applications are still limited by the availability of appropriate probes. For example, probes with sufficient specificity for prenatal diagnosis are not available for chromosomes 13 or 21. In addition, repetitive probes are not very useful for detection of structural aberrations since the probability is low that the aberrations will involve the region to which the probe hybridizes. Thus, most structural aberrations will be undetectable.

The development of procedures to construct probes of any desired specificity (e.g. to entire chromosomes or portions thereof) would advance a broad spectrum of cytogenetic studies. One approach is to use composite probes made from numerous sequences cloned from the desired target region. Recombinant DNA libraries for each of the human chromosomes, available from the American Type Culture Collection, have been constructed by the National Laboratory Gene Library Project(12). These can be used as starting material for chromosome-specific composite probes. The principal difficulty of using such libraries is that they contain both chromosome-specific sequences and sequences shared with other chromosomes. Therefore, the chromosome-specific sequences must be selected or hybridization

of the non-specific sequences must be blocked (21). We report here that competitive hybridization using entire chromosome-specific libraries for chromosomes 4 and 21 as probes and human genomic DNA as the competitor allows intense and specific fluorescent staining of human chromosomes 4 and 21 in metaphase spreads and interphase nuclei. Translocations and aneuploidy involving these chromosomes can be strikingly visualized. We also present data on hybridization with a collection of 120 unique-sequence probes on chromosome 4 to illustrate the possibility that true unique-sequence probes within the composite probes (as opposed to chromosome specific repeats) can produce the observed hybridization.

MATERIALS AND METHODS

Cells:

Metaphase spreads from human lymphocytes were prepared from methotrexate-synchronized cultures using the procedure of Harper *et. al.* (13). These and all other cells were fixed in 3:1 methanol/acetic acid. Other human lymphocyte cultures were irradiated with ^{60}Co gamma rays and stimulated with PHA. Colcemid was added 48 h after stimulation and metaphase spreads were prepared 4 h later. Metaphase spreads and interphase cells from

lymphoblastoid cells (GM03716A, Human Mutant Cell Repository, Camden NJ) carrying trisomy 21 were prepared after a 4 h colcemid block. Interphase cells from the cell line RS4;11 carrying t(4;11) and isochromosome 7 q were harvested, fixed in methanol/acetic acid, and dropped onto slides(14). Slides containing cells and chromosomes were stored at -20°C in plastic bags filled with nitrogen gas.

Probes:

Human chromosome-specific libraries produced by the National Laboratory Gene Library Project (12) were the source of all probes.

Chromosome 4 unique sequences: One hundred and twenty clones carrying chromosome 4-specific unique sequence inserts selected from the Charon 21A library LL04NS01 were supplied by Dr. C. Gilliam (15). The human inserts were all - 3 kb in length, so the ratio of insert to vector DNA was <0.1. Total phage DNA was produced from each clone individually. DNA pooled from 3 or all 120 clones was biotinylated by nick translation with biotin-11-d UTP (Bethesda Research Laboratories) and separated from the reaction mixture with Sephadex G-50 spin columns. DNA was recovered at a concentration of -20 ng/μl.

pBS-4: The entire chromosome 4 library LL04NS02 was subcloned into Bluescribe plasmids (Stratagene) to form the library pBS-4. The average insert to vector DNA ratio in pBS-4 is -1. The plasmid library was amplified in bulk and the DNA was extracted and biotinylated as described above. In some experiments, the biotinylated DNA was concentrated by ethanol precipitation to achieve higher probe concentrations.

pBS-21: The entire chromosome 21 library, LL21NS02, was subcloned into Bluescribe plasmids to form the library pBS-21. This library was amplified and biotinylated as described above.

Human Genomic DNA: Placental DNA (Sigma) was treated with proteinase K, phenol extracted and sonicated to a size range of 200-600 bp.

In situ **hybridization:**

Unique-sequence hybridization: Hybridization was accomplished using a modification of the procedure described by Pinkel *et. al.* (5). The slide mounted cells were treated with RNAse (100μg/ml in 2xSSC at 37°C for 1h), dehydrated in a 70% - 85%- 100% ethanol series, treated with proteinase K (-0.3 μg/ml in 20 mM tris; 2 mM CaCl2; pH 7.5 for 7.5 min at 37°C) and fixed (4%

paraformaldehyde in PBS plus 50 mM $MgCl_2$ for 10 min at room temperature). The DNA in the target cells was denatured by immersion in 70% formamide, 2X SSC, pH7 for 2 min at 70°C. The hybridization mix [10 µl total volume consisting of 50% formamide; 2xSSC (final concentration);10% dextran sulfate; 50 µg/ml sonicated herring DNA; and 3-6 ng of biotinylated chromosome 4 unique sequences (40-80 ng total phage DNA)] was then denatured (70°C for 5 min) and applied. Hybridization was at 37°C overnight (16 hr). Slides were washed in 3 changes of 50% formamide; 2xSSC (final concentration); pH7 at 45°C for 5 min each, and once in PN buffer (0.1M NaH_2PO_4 ; 0.1M Na_2HPO_4; 0.1% NP-40;pH8). The slides were then treated with alternating layers of fluoresceinated avidin-DCS and biotinylated goat-anti-avidin, both at 5µg/ml in PNM buffer (PN; 5% non-fat dry milk; 0.02% Na azide, centrifuged to remove solids) for 20 min each at room temperature until 3 layers of avidin were applied. The avidin-DCS and goat-anti-avidin treatments were separated by washes in PN. (Avidin and anti-avidin from Vector Laboratories). After the final avidin treatment, a fluorescence antifade solution (16) containing 1 µg/ml of DAPI or propidium iodide (PI) was applied to stain double stranded DNA (1.5 µl/cm^2 under a No. 1 coverslip).

Whole library hybridization: Hybridization was as above except that RNAse, proteinase K and paraformaldehyde were not used. The amount of probe and genomic DNA in the hybridization mix, and the length of the hybridization varied as described in Results. All probe concentrations refer to the human insert DNA unless otherwise noted. DNA concentrations were determined by fluorometric analysis (Hoeffer Scientific Instruments, San Francisco CA). Incubation of hybridization mix prior to hybridization followed two different protocols.

Protocol I: The hybridization mix contained 10 - 150 ng of biotinylated human DNA (20-300 ng of total plasmid DNA) and 0 to 10 μg of unlabeled genomic DNA. The mix was heated to denature the DNA and incubated at 37°C for a time T before it was added to the slide. Hybridization times ranged from 2 to 110 hours.

Protocol II: Identical to protocol I except that an additional aliquot of freshly denatured genomic DNA was added to the hybridization mix after an incubation time T. The mix was then incubated an additional time T prior to starting the hybridization. The volume of the hybridization mix was increased <20% by the additional genomic DNA.

Microscopy:

Slides were viewed and photographed using either a Zeiss Universal microscope with camera or a Zeiss Axiophot. Quantitative fluorescence measurements were performed using a video camera on the microscope and a digital image processing system (4). Photography was on Kodak Ektachrome 400 transparency film. The combination of red PI and green-yellow FITC fluorescence appears as yellow in the photographs.

RESULTS

Whole library hybridization:

Figure 1a shows hybridization of pBS-4 to a human metaphase spread with a probe concentration of 1 ng/l. No genomic DNA was used and the hybridization mixture was applied immediately after denaturation. The inclusion of unlabeled total human DNA in the hybridization mixture substantially increased the specificity of hybridization to chromosome 4. Figures 1b and 1c show the result of a protocol I hybridization [0.8 ng/l probe, 24 ng/l genomic DNA (Q = 2, see Discussion), 1 h probe incubation 110 h hybridization]. This probe concentration corresponds to approximately

$0.5x10^{-2}$ pg/μl/kb for unique sequences, assuming that 30% of the chromosomal DNA is unique. Quantitative image analysis shows that the intensity per unit length of chromosome 4 is approximately 20 times that of the other chromosomes. This is referred to as the contrast ratio. Two layers of avidin-FITC have been used here to make the non-target chromosomes sufficiently bright to be measured accurately. However, the number 4 chromosomes can be recognized easily after a single layer. Hybridization for shorter periods also allows selective staining of chromosome 4, but with somewhat lower contrast. Shorter hybridizations (16 h) are used routinely for aberration detection.

Figure 1d demonstrates detection of a radiation-induced translocation involving chromosome 4 in human lymphocytes [protocol I, 1 ng/μl probe, 76 ng/μl genomic DNA (Q = 5, see Discussion), 1 h probe incubation, 16 h hybridization]. The contrast ratio was ~5. Fig. 1e shows that the normal and two derivative chromosomes resulting from the t(4;11) in cell line RS4;11 can be detected in interphase nuclei as three distinct domains [protocol I, 13.5 ng/μl probe, 800 ng/μl genomic DNA (Q = 5, see Discussion), 1 h probe incubation, 16 h hybridization]. The increased probe

concentration resulted in brighter signals relative to Fig. 1d. Approximately half the cells clearly show the presence of three nuclear domains.

Hybridization of pBS-21 to a metaphase spread from a cell line with trisomy 21 is shown in Fig. 1 f [protocol II, 4 ng/μl probe, 250 ng/μl genomic DNA, 3 h incubation, additional 250 ng/μl genomic DNA (Q = 1+ 1, see Discussion), 3 h probe incubation, 16 h hybridization]. A small amount of hybridization is visible near the centromeres of the other acrocentrics. Fig. 1g shows two interphase nuclei from this same hybridization. These clearly show the 3 chromosome 21 domains. Hybridization with probe prepared according to Protocol I resulted in higher relative intensity of the shared signals on the D- and G-group chromosomes, and consequently it was more difficult to determine the number of number 21 chromosomes in interphase (not shown). Increasing stringency by using a hybridization mix consisting of 55% formamide; 1xSSC, which lowers the melting temperature about 8°C, did not reduce the unwanted hybridization. Addition of unlabeled pA ribosomal DNA (17) also was ineffective at increasing specificity.

Unique sequence hybridization:

Hybridization with a pool of 3 chromosome 4 unique sequences was conducted at a total phage concentration of 2 ng/μl per clone, corresponding to 50 pg/μl/kb of human insert. Individual hybridization sites could be seen (not shown) after overnight hybridization (16 h) and application of three layers of avidin. Analysis of 45 spreads in two experiments showed 265 fluorescent spots on chromosome 4 out of the 540 (6 per chromosome) possible, indicating a hybridization efficiency of -50%. The total number of spots on all chromosomes was 568, giving a hybridization specificity of -50%. The result of a 16 h hybridization of a collection of 120 unique sequence probes is shown in Figure 1h. Note that individual hybridization sites can be seen and located to within a fraction of the width of a chromatid. The total probe concentration for this hybridization was the same as for the group of 3, resulting in -1.5 pg/μl/kb of human insert. Analysis of 3 spreads showed 222 fluorescent spots out of the 1440 maximum possible on the number 4 chromosomes, yielding a hybridization efficiency of 15%. Eight hundred fourteen total spots were found on all the chromosomes, giving a specificity of 27%. These experiments suggest that

substantial hybridization can occur with unique sequence probes at low probe concentrations.

DISCUSSION

Competitive hybridization

This paper describes the use of unlabeled genomic DNA to competitively inhibit hybridization of those sequences in a chromosome-specific library that are shared with other chromosomes. This produces intense, specific staining of the selected chromosome type in metaphase spreads and interphase nuclei. Development of these protocols required optimization of a number of factors including: the amounts of probe and blocking DNA, the probe incubation time(s) prior to hybridization, and the time and conditions of hybridization. The following sections present a semi-quantitative discussion of the considerations that led to the protocols reported in this paper. The Appendix contains the mathematical details. Prehybridization of the target cells with genomic DNA was also explored, but was found to be unnecessary for high contrast ratio hybridization and will not be discussed further.

Hybridization without genomic DNA: Fig. 1a demonstrates the hybridization of pBS-4 DNA to a human metaphase spread. All of

the chromosomes are stained along most of their lengths due to sequences in the probe that are shared with other chromosomes. Unstained regions, noted by arrows, show locations for which homologous sequences are not present in pBS-4. These are mostly in the centromeric regions and the long arm of the Y, where blocks of repetitive DNA specific to those sites are known to exist. The visible contrast on chromosome 4 is the result of the interaction of several factors: 1) all of the DNA in chromosome 4 is potential target for sequences in the probe, while only those sequences on the other chromosomes that are shared with chromosome 4 can bind probe. 2) The hybridization time and probe concentration were high enough to allow significant binding of the specific sequences. 3) The ratio of probe to target sequences is higher for the specific sequences than for the shared sequences. (Ten ng of chromosome 4 DNA was hybridized to - 200 ng of human DNA target ($4x10^4$ cells), 13 ng of which is chromosome 4. Thus, the ratio of probe to target for the specific sequences was - 1, while for the shared sequences it was - 0.05).

The contrast can be increased by allowing the probe DNA to partially reanneal prior to adding it to the slide. This preferentially depletes the high-copy (predominantly the shared) sequences in the

probe because the reassociation rate of a sequence is proportional to its concentration, eq. A1. Fig. 2a (curves labeled Q = 0) shows the calculated renaturation of a chromosome-specific and a highly repeated, shared sequence. The concentration of the single-stranded high-copy sequence is reduced almost a factor of 1000, during the same period that the concentration of the single stranded specific sequence is reduced a factor of 2. A significant increase in hybridization specificity resulting from probe reannealing prior to hybridization was observed experimentally for chromosome 4 using a hybridization mix with 1 ng/μl of probe and a prehybridization incubation at 37°C for 24 h (not shown). Likewise, a 24 h incubation of 4 ng/μl of chromosome 21 probe resulted in a substantial contrast ratio. Thus, at these concentrations, labeled, chromosome-specific sequences remain single stranded for days in hybridization mix indicating that other mechanisms that might inactivate the probe are not operating.

Protocol I Hybridization: The addition of unlabeled genomic DNA to the hybridization mix increases the concentration of the shared sequences by a larger factor than it increases the concentration of chromosome specific sequences. This enhances the preferential reassociation of the labeled copies of the shared

sequences and increases the contrast ratio. To see this, first consider one of the sequences, repeat or unique, that is specific for the i^{th} chromosome in a hybridization mix containing a mass m_p of probe DNA from the i^{th} chromosome library and m_b of unlabeled genomic DNA. The number of labeled copies of the sequence is proportional to m_p. However, the number of unlabeled copies is proportional to $f_i m_b$, where f_i is the fraction of genomic DNA contained on the i^{th} chromosome. Thus, Q, the ratio of unlabeled to labeled copies of each of the sequences specific for the target chromosome, is $f_i m_b/m_p$. For human chromosomes, $0.016 < f_i < 0.08$ (18). For the work reported in this paper, $f_4 = 0.066$ and $f_{21} = 0.016$. Now consider a shared sequence that is distributed more-or-less uniformly over the genome. The number of labeled copies is proportional to m_p, while the number of unlabeled copies is proportional to m_b. Thus, the ratio of unlabeled to labeled copies is $m_b/m_p = Q/f_i >> Q$. This is true for all uniformly distributed sequences, regardless of copy number. Thus adding genomic DNA increases the concentration of each specific sequence by the factor 1 + Q, while each uniformly distributed sequence is increased by the factor $1 + Q/f_i$. Figure 2a compares reassociation of the specific and shared sequences with Q = 2 of

genomic DNA to case with the Q = 0 discussed previously. Note that the concentration of labeled single-stranded DNA of the distributed sequence is decreased much more dramatically relative to the Q = 0 curve than is the specific sequence. In fact it is driven to be less than that of the specific sequence. The mathematics describing this are in equations A2 and A3.

While use of some genomic DNA is necessary to generate a high contrast ratio, use of too much will decrease the intensity of the specific staining to unacceptable levels. This is due to reassociation of the labeled specific sequences in the probe and competition of the labeled and unlabeled copies for hybridization sites. It can be shown, eq. A2 and A3, that roughly half of the beneficial effect of genomic DNA on relative renaturation rates is achieved when Q = 1, and by Q = 5, there is essentially no more benefit to be gained. Thus, our protocol I hybridizations keep $Q < 5$.

Results also improve if the hybridization time is sufficiently long to give the specific sequences the opportunity to find their binding sites. Since there are more binding sites available for the high-copy (shared) sequences than for the specific sequences, staining of the non-target chromosomes occurs more rapidly. Thus contrast improves as hybridization time is extended. While we have

no direct evidence concerning the type of sequence predominantly responsible for the chromosome-specific signals, Fig. 1h indicates that we do have the sensitivity to detect unique sequence hybridizations of 3 kb target sites. However, low-copy chromosome-specific repeats, such as VNTRs (19), may contribute to the hybridization as well. If the unique sequences are responsible for the specific signals, then the concentration of each sequence in our chromosome 4 hybridizations is about 10 to 100 times lower than that used in Fig. 1h. Thus one might expect that hybridization times greater than 16 h would be beneficial.

Figs. 1b and 1c illustrate that very high contrast can be achieved by following these guidelines. A contrast ratio of ~20 was achieved on chromosome 4 using 1 ng/μl probe, Q = 2 of genomic DNA, 1 h probe incubation and a 110 h hybridization. Weaker, but still adequate signals with a contrast ratio of ~ 5 are achieved with a 16 h hybridization under these conditions. Very bright signals can be achieved with a 16 h hybridization using 13.5 ng/μl of probe and Q = 5, Fig. 1e. The contrast is reduced relative to that achieved in Fig. 1b, but it is more than adequate. Experiments with increasing amounts of carrier DNA indicate that results become worse as the

total DNA concentration approaches 1 μg/μl, so our protocols stay below this limit.

Protocol II hybridization: Hybridization with pBS-21 DNA using protocol I presents a problem not encountered with chromosome 4. This DNA hybridizes both to chromosome 21 and to the centromeric regions of the D- and G-group chromosomes. These regions contain ribosomal(17) and alpha satellite (20) sequences and perhaps others. These are relatively low-copy sequences shared with only a few chromosomes, so protocol I is not very effective at suppressing them relative to the 21-specific sequences. In addition these sequences are clustered on the chromosomes, so that even much reduced hybridization is clearly visible. This is especially distracting in analysis of interphase nuclei. Calculations (eq. A4 and A5) illustrated in Fig 2b indicate that addition of several aliquots of freshly denatured genomic DNA periodically during the incubation should be much more effective than protocol I at reducing the concentration of labeled single-stranded DNA from the acrocentric repeats relative to the specific sequences. Fig. 1f shows a protocol II hybridization to a metaphase spread from a trisomy 21 cell line. Intense hybridization to the three number 21 chromosomes is clearly visible and hybridization to the other D- and G-group chromosomes

has been reduced to an acceptable level. Fig. 1g shows that hybridization to chromosomes other than 21 is sufficiently low that the three chromosome 21 domains are clearly visible in interphase nuclei. In practice, the most convenient procedure for suppressing the shared acrocentric hybridization might be inclusion of unlabeled DNA from one of the other D- or G-group chromosome libraries as additional competitor.

Aberrant chromosome detection

Detection of numerical and structural aberrations with composite probes should facilitate many areas of cytogenetics. Staining with the composite probe for chromosome 4 permits rapid identification of translocations in metaphase spreads, Fig. 1d. The hybridization intensity and specificity is such that even very small portions of the involved chromosome can be detected. This technique should be especially useful for applications where high quality banding is difficult (e.g. in tumor cytogenetics) or where speed of analysis is the predominant concern (e.g. for detection of low frequency induced chromosomal aberrations). The high contrast staining may eventually permit the use of automated analysis techniques. Fig. 1e demonstrates the detection of a homogeneously

occurring translocation involving chromosome 4 in interphase nuclei from a leukemia cell line (14) carrying the translocation t(4;11). Approximately half of the nuclei of this cell line showed three brightly fluorescent domains, presumably produced by the two portions of the derivative chromosome 4 and the intact normal chromosome. The domains in the other nuclei may have been obscured by the nuclear orientation in these two dimensional views, by nuclear distortion that occurred during slide preparation, or because the domains were too close to each other to be distinguished. Hybridization using procedures that preserve three dimensional morphology may resolve these issues, and also permit general studies of chromosomal domains in interphase nuclei (4, unpublished).

Prenatal screening for disease-linked chromosome aberrations (e.g. trisomy 21) would be enhanced by techniques that allow more rapid detection of such aberrations. Figures 1f and 1g show that competitive hybridization with pBS-21 DNA allows detection of trisomy 21 in metaphase spreads and in interphase nuclei. Interphase aneuploidy analysis is particularly exciting since it will yield rapid results (cell culture is not necessary). Whether the hybridization patterns are sufficiently reliable for routine prenatal diagnosis remains to be determined. Complications may result from clustering,

overlap, and/or distortion of the fluorescent domains. However, these preliminary results are encouraging.

Chromosome-specific libraries for all of the human chromosomes are now available, so that production of a kit of reagents capable staining each of the human chromosomes should be straightforward. The procedure also should work for other species whose genomes have the same general features as the human. Staining of subchromosomal segments should be possible using libraries containing those sequences. Large insert libraries, cosmids and eventually yeast artificial chromosomes, are being produced for the human chromosomes. These will facilitate selection of probes for chromosomal segments since single clones from these libraries can be hybridized to human chromosomes and nuclei with efficiencies approaching 100% (21, Landegent in prep).

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APPENDIX

Reassociation during probe incubation:

Protocol I. Let C_o be the initial concentration of single-stranded copies (labeled and unlabeled) of one of the (many) sequences in the hybridization mix. If the mixture is incubated at a temperature that allows reassociation of the complimentary strands, the concentration C(t) of single-stranded copies of the sequence remaining at time t is

$$C(t) = C_o/(1 + kC_ot) \qquad \text{Eq. A1}$$

where k is the rate constant (22). We define the ratio of labeled to total single-stranded copies of the sequence as the competition factor $r = C_p(t)/C(t)$, where $C_p(t)$ is the concentration of the labeled copies. r is independent of time if labeled and unlabeled copies of the sequence behave indistinguishably. Then:

$$C_p(t) = C_{op}/(1 + kC_{op}t/r) \qquad \text{Eq. A2}$$

where C_{op} is the initial concentration of labeled single-stranded copies. Note that the presence of the genomic DNA increases the effective rate constant from k to k/r. The value of r depends only on

how the copies of that sequence are distributed in the genome. r is of the form:

$$r = 1/(1+\beta) \qquad \text{Eq. A3}$$

Following the discussion in the paper, $\beta = Q$ for chromosome-specific sequences, Q/f_i for uniformly distributed sequences, and $5Q$ for sequences shared equally by all the D- and G-group chromosomes. Sequences with other patterns of distribution over the genome have other values of β.

Protocol II. If the genomic DNA is divided into two equal aliquots, one included initially with the probe and the other added after an incubation time T, the competition factor R, analogous to eq. A3 is:

$$R = \frac{[\ r\ ]}{2-r}\ \frac{[\ 1\ ]}{1 + [(1-r)/(2-r)]kC_{op}T/r} \qquad \text{Eq. A4}$$

where r is from eq. A3 for the first incubation period. The first factor gives the value of R if all the genomic DNA had been added at the beginning of the incubation, e.g. protocol I. The second reflects the sequence reassociation between the addition of the two aliquots. As the incubation is extended after the second addition of blocking DNA, reassociation continues. If the second aliquot of genomic DNA

does not substantially change the volume of the hybridization mix, the concentration $C_p(t)$ of labeled single stranded copies of a sequence at time $t > T$ is:

$$C_p(t) = \frac{C_{op}}{1 + kC_{op}T/R + kC_{op}(t = T)/R} \qquad \text{Eq. A5}$$

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FIGURE CAPTIONS:

Figure 1.

a) Hybridization of pBS-4 DNA with no genomic DNA to a human metaphase spread. Arrows indicate regions that are unstained with the probe.

b) Hybridization of pBS-4 to a human metaphase spread with Q = 2 of genomic DNA. Chromosome 4 is yellow, others are red due to the PI counterstain.

c) Same spread as b) but using a filter that passes only the FITC fluorescence.

d) Detection of a radiation induced translocation (arrows) involving chromosome 4 in a human metaphase spread.

e) Representative nuclei from cell line RS4;11 which homogeneously contain t(4;11)

f) Hybridization of pBS-21 to a metaphase spread of a trisomy 21 cell line.

g) As f) but interphase nuclei.

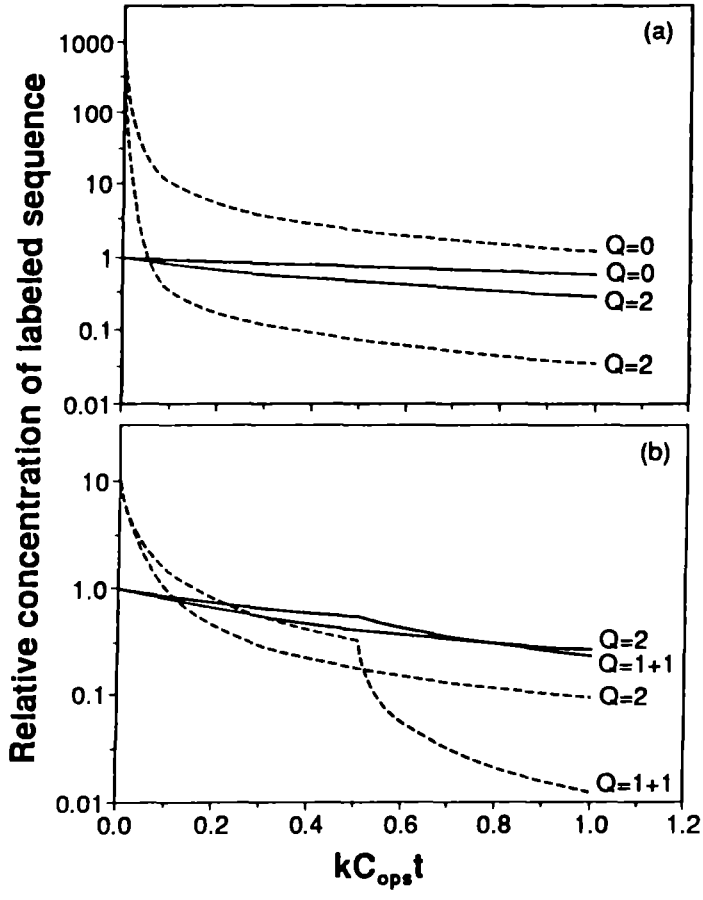
h) Hybridization of 120 unique-sequence probes from chromosome 4 to a human metaphase spread.

Figure 2.

Relative concentration of labeled single-stranded copies of DNA sequences during prehybridization incubation calculated as described in the appendix.

a) Protocol I, chromosome 4: The solid curves show the time dependence of the concentration of labeled single-stranded copies of a chromosome-specific sequence during an incubation without genomic DNA (Q = 0) and with genomic DNA (Q = 2). The dotted curves show the same for a shared sequence that is uniformly distributed over the genome and that has 1000 times as many copies as the specific sequence on chromosome 4. Time is measured in units of kC_{ops}, where k is the rate constant and C_{ops} the initial concentration of labeled single stranded copies of the chromosome-specific sequence.

b) Comparison of Protocol I and Protocol II, chromosome 21: The solid curves compare the behavior of a chromosome-specific sequence incubated according to protocol I using Q = 2 of genomic DNA, with protocol II using Q = 1 of genomic DNA and an additional Q = 1 half way through the incubation. There is almost no difference in the concentrations at the end of the incubation. The dashed curves compare these same two procedures for a sequence shared equally among the D- and G-group chromosomes and that has 10 times as many copies on chromosome 21 as the specific sequence. The concentration of the shared sequence is much more effectively reduced using protocol II.


(a)
1000
100
10
1
0.1
0.01
Q=0
Q=0
Q=2
Q=2
(b)
10
1.0
0.1
0.01
Q=2
Q=1+1
Q=2
Q=1+1
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Relative concentration of labeled sequence
kC$_{ops}$t

a
b
c
d
e
f
g
h